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THE EFFECT OF IODINE AND IODOTHYRINE ON THE LARVÆ OF SALAMANDERS. II. THE RELATION BETWEEN METAMORPHOSIS AND LIMB DEVELOPMENT IN SALAMANDER LARVÆ.

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In previous experiments (1) on the larvæ of *Ambystoma opacum* I found that iodothyrine did not accelerate the development of the limbs, although it caused rapid metamorphosis. Consequently, if the administration of iodothyrine was begun at an early larval stage, the metamorphosed salamanders possessed hind limbs which did not have the full number of toes. In agreement with these observations is the fact that feeding of thymus gland, although it resulted in an inhibition of metamorphosis, did not retard the development of the limbs of the thymus-fed salamander larvæ (2). Hence it is evident that in salamander larvæ the development of the limbs is independent of the substance (thyroid hormone) which causes metamorphosis.

The relation between limb development and metamorphosis as it exists in salamander larvæ is of especial interest, since apparently it is just the opposite of what should have been expected from the experiments performed on the anuran tadpoles. Through the work of Gudernatsch (3) and many other investigators it is well known that in tadpoles administration of thyroid gland, iodothyrine and other thyroid preparations accelerates not only metamorphosis, but also the development of the limbs. Lately Swingle (4) found that the administration of inorganic iodine which causes precocious metamorphosis of the tadpoles likewise accelerates development of the limbs.

Recent investigations have shown that the thyroid hormone and probably other morphogenic hormones, by increasing the rate of certain fundamental reactions, have the ability of causing structural changes throughout the entire organism, bringing thus about morphological expressions of a wide range affecting nearly the whole body. It seems that these hormonic substances, as far as their immediate effect is concerned, act chiefly by inducing a general histolysis throughout the various organs of the organism. There can be no doubt, however, that, besides these hormones referred to above, other substances play an important rôle in the development of an organism; these substances seem to possess a more localized action, effect the development of only certain organs and are concerned chiefly with the building up of the structures of these organs. Certainly, in the evolution of the organisms, the acquirement of the ability of elaborating the latter kind of substances must have played a rôle equally important as that played by substances such as the thyroid hormone. The limbs of the amphibians are apparently organs whose development seems to depend chiefly on the action of substances belonging to the latter group of substances and not on the activity of the thyroid hormone.

On account of the increased importance which, in the light of such considerations, seemed to attach itself to the finding that the development of the salamander limbs is independent of the thyroid hormone, it appeared necessary to repeat my previous experiments on the relation between limb development and metamorphosis. The present article will be devoted to reporting these new experiments. They consisted in causing precocious metamorphosis of the larvæ of *Ambystoma maculatum* by keeping them in iodothyrine, whereby special attention was paid to a possible acceleration of limb development. The result was the same as in the experiments on *A. opacum;* the rate of the development of the limbs remained unchanged, although metamorphosis took place at an early date. Not only larvæ, but also embryos, at early stages, were exposed to the influence of iodothyrine, in order to avoid the objection that failure of the iodothyrine to cause accelerated limb development was due to the experiments having been started at a stage at which limb development was too far advanced. Again

these experiments were completely negative as to an acceleration of limb development. In order to be certain that the method employed in my experiments on salamanders was correct, several tests were carried out on tadpoles; these were positive.

In addition to these experiments, several experiments were made to test the influence of inorganic iodine on the development of the amphibian limbs. Like Swingle (4), I found a distinct acceleration of the limb development in tadpoles; in the salamander larvæ, however, iodine had no effect on the development of the limbs. The bearing of this result, which is different from that of the experiments with iodothyrine will be referred to in the discussion.

Experiments on Tadpoles.

The experiments on tadpoles, which were intended to serve as a check to the experiments on salamanders, were not advanced beyond a merely preliminary stage, since they gave positive results from the very beginning. As they are confirmative of the observations reported by other investigators, they will be reported only briefly.

In one experiment on the larvæ of *Rana sylvatica* the controls were kept under observation till the 66th day after hatching. At this time the hind limbs of the control larva furthest advanced possessed 3 distinctly differentiated toes, while the 2 other toes were still rudimentary. In the iodothyrine series (kept from the 18th to the 26th day in water which contained 0.005 gm. Bayer's iodothyrine per 1,000 c.c. of water) the fore limbs broke through the walls of the gill chamber on the 33d day after hatching in every one of the 3 larvæ surviving, at this date, from the 6 larvæ composing the series at the beginning of the experiment. The inorganic iodine, in the concentration used in this experiment (2 to 3 drops of a 1/20 M solution of iodine per 1,000 c.c. of water), as well as in other experiments, proved to be considerably less effective than the iodothyrine, as 66 days after hatching the fore limbs had not perforated the gill chamber in a single instance. Yet the limbs, the hind limbs as well as the fore limbs, were distinctly further differentiated than in the controls of the same age; the hind limbs possessed 5 fully differentiated toes and in shape

were much like the hind limbs of an adult frog. Moreover, in 2 larvæ of the iodine series, which died at an age of 50 and 56 days respectively, the hind limbs possessed already at that date 5 fully differentiated toes.

In another experiment 5 series of the tadpoles of *Rana sylvatica* were kept in different concentrations of iodine (varying from 1 to 10 drops of a 1/20 M solution of iodine per 1,000 c.c. of water). The controls were kept under observation for 83 days; at the end of this period the hind limbs had remained undifferentiated, whitish buds in 5 of the 8 larvæ, while in the other 3 larvæ differentiation had taken place, the best differentiation being represented by 4 toes on the foot of the hind limbs. Many of the larvæ kept in inorganic iodine died; none of these was further advanced than the surviving larvæ. Among the surviving larvæ none had metamorphosed at the termination of the experiment, nor had the fore limbs broken through in a single instance; yet the limbs were considerably further differentiated than in the controls. In one larva of the iodine series, at an age of 73 days, the foot of the hind limbs was differentiated into 5 toes; in another larva, at an age of only 59 days, the hind limbs possessed 5 toes, and the fore limbs, which could be seen vigorously moving in the gill chamber, had developed 3 toes.

There can be no doubt that iodothyrine when administered to tadpoles greatly accelerates development of the limbs. Inorganic iodine, although it seemed less efficient in these experiments than iodothyrine, likewise increases the rate of the development of the limbs.

Experiments on Salamander Larvæ.

As pointed out in the introduction, my previous experiments on the larvæ of *Ambystoma opacum*, in which the administration of iodothyrine did not accelerate development of the limbs, were open to the criticism that the administration of iodothyrine was started at a stage at which limb development was fairly advanced (the toes having begun to differentiate), and that for this reason the iodothyrine may have been incapable of accelerating limb development. Therefore it seemed necessary to start the experiment at a very early stage. Two experiments were carried out, both on the embryos of *Ambystoma maculatum*.

Experiment I.—One egg mass of *Ambystoma maculatum* was collected on April 18, 1920. The eggs were not only freed from the general mass of jelly, but also the individual egg envelopes were removed, in order to assure ready access of the iodothyrine and iodine to the developing embryos.

Beginning of the experiment: 28 embryos selected; 9 of them placed into iodine-free water (10,000 c.c. H_2O, 0.16 gm. Na_2CO_3, 0.04 gm. K_2CO_3, 0.4 gm. $MgSO_4$–$7H_2O$, 0.6 gm. $CaCl_2$), 9 into iodine (2 drops of a 1/20 M solution of inorganic iodine per 1,000 c.c. of iodine-free water) and 10 into iodothyrine (0.01 gm. Bayer's iodothyrine per 1,000 c.c. of iodine-free water). In all embryos the first four visceral arches are formed; the fore-limb rudiments not yet differentiated from the pronephridial protuberance; no hind limbs.

The concentration of the inorganic iodine was increased to 8 drops per 1,000 c.c. 5 days, decreased to 6 drops 7 days, and decreased to 4 drops 11 days after the beginning of the experiment.

Sixteen days after the beginning of the experiment: Neither the iodine nor the iodothyrine had produced any influence on the development of the limbs (see Table I.).

TABLE I.

EXPERIMENT I.: 16 DAYS AFTER BEGINNING OF EXPERIMENT.

	Total Number.	Development of Toes.		
		3.5	3.0	2.5
Water	8	7		1
Iodine	8	5	1	2
Iodothyrin	10	9		1

Twenty days after the beginning of the experiment: The concentration of the iodine is decreased to 3 drops per 1,000 c.c. of water, the concentration of the iodothyrine increased to 0.1 gm. per 1,000 c.c. of water.

Twenty-seven days after the beginning of the experiment: Hind limbs commenced to differentiate into toes; but neither iodothyrine nor iodine accelerated limb development as compared to limb development of controls kept in iodine-free water (see Table II.).

Table II.

Experiment I.: 27 Days after Beginning of Experiment.

	Total Number.	Development of Toes.		
		6.0	4.5	4.0
Water	8	3	5	
Iodine	7		4	3
Iodothyrin	10		2	8

Yet the influence of the iodothyrine on metamorphosis had become noticeable in spite of the early stage of the larvæ, as the gills as well as the fin of the tail are found to be greatly atrophied.

Experiment II.—In this experiment one series of the embryos of *Ambystoma maculatum* was kept in inorganic iodine and one in iodine-free water. The concentration of iodine was 1 drop of a 1/20 M iodine solution per 1,000 c.c. of iodine-free water in the beginning, was increased later on to 8 drops and then gradually decreased to 3 drops. The embryos were at an early stage (formation of neural folds), when the experiment started; only the common jelly mass was removed.

Table III.

Experiment II.: 10 Days after Beginning of Experiment.

	Total Number.	Fore Limb Buds Present, No Indication of Toes.
Water	15	15
Iodine	14	14

Table IV.

Experiment II.: 18 Days after Beginning of Experiment.

	Total Number.[1]	Development of Toes.	
		3.5	2.5
Water	14	14	
Iodine	13	7	6

[1] Several eggs and embryos were attacked by moulds and as they disintegrated or developed abnormally, they had to be discarded causing thus the decreases in the total numbers.

TABLE V.

EXPERIMENT II.: 30 DAYS AFTER BEGINNING OF EXPERIMENT.

	Total Number.[1]	Development of Toes.		
		6.0	4.5	4.0
Water	14	5	7	2
Iodine	12	1	10	1

As Tables III., IV. and V. show, the inorganic iodine had no influence whatsoever on the development of the limbs.

The relation between limb development and metamorphosis was further tested in two experiments in which larvæ of *Ambystoma maculatum* at a more advanced stage were employed. Concerning the action of iodothyrine, the results were in complete accordance with those obtained in the larvæ of *Ambystoma opacum;* rapid metamorphosis, but no influence on limb development was observed. In each experiment one series was devoted to the study of the influence of inorganic iodine; this substance likewise had no influence on limb development, but unlike iodothyrine it did not cause precocious metamorphosis. Both iodine experiments as regards the influence of iodine on metamorphosis were described in detail in a previous article (5); they will be only briefly reported in this article.

In Experiment III. the larvæ were placed into iodine-free water containing 0.1 gm. iodothyrine per 1,000 c.c. of water at an age of 20 days, at which date nearly all larvæ had developed 4 toes in the fore limbs and several had commenced to develop the first 2 toes in the hind limbs. Thirty-three days after hatching—*i.e.,* 13 days after the first administration of iodothyrine—every one larva metamorphosed (as compared to 101 days in the controls), but in none of them the number of toes was more than 7.5, and in one it was only 6.0, this stage of limb development corresponding to the control series kept in iodine-free water without the addition of iodothyrine.

In Experiment IV. a smaller dosis of iodothyrine (0.01 gm. per 1,000 c.c. of iodine-free water) was administered. Precocious metamorphosis was caused also by this dosis, but the development of the limbs again remained completely unaffected as compared to

the controls. Since, however, metamorphosis took place at an age at which normally the limbs are fully developed (as shown by the controls), the precociously metamorphosed salamanders possessed in this experiment fully developed limbs.

Concerning the influence of inorganic iodine in these experiments, it was shown in a previous article (5) that administration of iodine does not result in precocious metamorphosis of salamander larvæ. In this article it should be added that it did have no effect also on the development of the limbs.

Discussion.

The experiments reported in this article confirm fully the observations made in my previous experiments. In the larvæ of salamanders the development of the limbs can not be accelerated by the administration of iodothyrine. Therefore, if iodothyrine is administered in doses which cause metamorphosis before the time at which, under normal conditions, the limbs are fully developed, metamorphosis takes place before the completion of limb development.

These facts demonstrate that in salamanders limb development is independent of the substance (thyroid hormone) which causes metamorphosis. This conclusion has recently been supported by several other facts. *Typhlomolge rathbuni,* the Texan cave salamander, does not possess a thyroid gland (6) and consequently does not metamorphose; yet its limbs develop in a normal manner. Hoskins and Hoskins (7) have shown that in the larvæ of *Ambystoma* the limbs develop normally, if the larvæ are deprived of their thyroid glands in early embryonic stages. This season I have repeated these experiments. Larvæ of *Ambystoma maculatum* were thyroidectomized at an early embryonic stage; these larvæ which are believed to have been successfully operated on (histological examination has not been made as yet) showed the same rate of limb development as the controls. Several larvæ of *Ambystoma tigrinum* were thyroidectomized at a stage at which 3 toes of the hind limbs were developed; the two other toes developed at a normal rate after thyroidectomy. These facts prove that in salamanders the substances causing limb development are not identical with those causing metamorphosis and consequently are not identical with the thyroid hormone.

In tadpoles substances which cause precocious metamorphosis also accelerate the development of the limbs. I have suggested, in a previous article (8), that in spite of this external difference existing between the larvæ of *Anura* and *Urodela* the primary effect of the thyroid hormone may be the same in both groups of animals, and the difference may not be a fundamentally different reaction upon the thyroid hormone, but merely a different mode of limb development.

There is no doubt that, except for the development of the limbs in tadpoles, the immediate effect of the thyroid hormone is, in both groups, predominantly a breaking down of tissues throughout the whole organism, not a building up of new organs. It is possible that in tadpoles the same substances endowed with a merely localized action cause limb development as in salamanders, but that in tadpoles these substances can not commence to build up the structures of the limbs before some obstacle has been cleared away by the action of the thyroid hormone. That the thyroid hormone controls limb development in the tadpoles does not necessarily mean that it has any part in the constructive processes of limb development. If we consider the advanced stages of the development of the fore limbs in tadpoles, we find conditions which make it indeed very probable that the thyroid hormone, in this process, plays merely the rôle of removing an obstacle external to the tissues of the limb itself. In salamanders both hind and fore limbs develop freely, while in tadpoles the fore limbs are inclosed in the gill chamber. In order that they may break through the walls of the gill chamber, certain changes of the skin and the tissues underlying it must take place; these changes are not caused by the legs themselves, but take place even in the absence of the limbs (9) at the time at which metamorphosis occurs. I have pointed out repeatedly that in salamanders one of the most conspicuous effects of the thyroid hormone is a certain change of the skin which finally results in the shedding of the skin and may be identical with the process which leads to the atrophy of the gills. A similar change is brought about in the skin of the tadpoles; in the tadpoles, too, the skin is shed for the first time when metamorphosis takes place. It is possible that the change of the skin which is necessary to

permit of the freeing of the fore limbs is identical with the change that causes the first shedding of the skin.

In support of this view is the fact that the freeing of the fore limbs is clearly the only step in the development of the limbs, which in not one single instance has been observed to take place in tadpoles which had been deprived of the thyroid secretion, while the developmental processes preceding the freeing of the limb may take place in thyroidectomized tadpoles. Allen (10) states that in tadpoles of *Bufo*, in the complete absence of a thyroid, both fore and hind limbs develop normally (and even grow larger than the limbs of normal larvæ)—*i.e.*, behave exactly like the limbs of salamander larvæ—yet the fore limbs fail to break through the walls of the gill chamber. Apparently in this anuran species the development of the limbs, except for the freeing of the fore limbs, is independent of the thyroid hormone as it is in the urodelan larvæ.

Should these views prove to be correct, it would seem probable that the mode of limb development in amphibians is the morphologic expression of the existence of two kinds of morphogenic substances; one group of these substances serves to procure the actual building stones of the morphological structures of the organ, while the other group of substances merely brings about a general histolysis of old structures, removing thus obstacles to the action of the substances belonging to the first group of substances.

As has been mentioned above, the ineffectiveness of inorganic iodine in the limb development of salamanders has a reason different from that of the ineffectiveness of iodothyrine. In a previous article (5) I have shown that, in contradistinction to iodothyrine, the administration of inorganic iodine does not produce precocious metamorphosis in salamander larvæ. The inorganic iodine has no effect on the salamander metamorphosis, because the thyroid hormone in salamanders is not released during the greater part of the larval period, and a greater supply of inorganic iodine, even if it should result in the elaboration of an excess of thyroid hormone, as it actually does in tadpoles (11), can not make itself felt in the salamander larvæ in consequence of the retention of the hormone. As has been shown in this article, inorganic iodine, like iodothyrine, has no effect on limb development of salamanders. But it must

be borne in mind that the ineffectiveness of inorganic iodine in limb development of salamanders is merely due to the above-mentioned peculiarity of the thyroid apparatus of the salamander larvæ and not to the fact that limb development in salamanders is independent of the thyroid hormone and of metamorphosis. Inorganic iodine could not accelerate limb development of salamanders, even if iodothyrine would have an accelerating effect.

Summary.

1. Tadpoles of *Rana sylvatica* were kept in iodothyrine and in solutions of inorganic iodine. Both substances were found to accelerate limb development. This result confirms the observations of previous investigations.

2. Embryos as well as larvæ, in early stages, of *Ambystoma maculatum* were kept in iodothyrine and in inorganic iodine. Neither of these substances accelerated the development of the limbs, although iodothyrine caused rapid metamorphosis of the salamander larvæ.

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